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**13. ABSTRACT (Maximum 200 Words)**

Carcinogen-treated rats that are mated and produce litters will develop fewer mammary cancers than if they had remained virgin. This constitutes a model for studying how parity reduces breast cancer risk for women. Employing the model, investigators have given pregnancy-associated hormones in lieu of pregnancy to carcinogen-treated rats to assess their cancer-inhibiting capability. Investigators gave estriol ($E_3$), $E_3$ plus progesterone ($P_4$), estradiol ($E_2$) plus $P_4$, human chorionic gonadotropin, and (by us) alphafetoprotein (AFP). Perplexingly, all the treatments reduced breast cancer incidence. Out purpose is to test the hypothesis that each of the hormone treatments elicited AFP from the adult liver, which has been the proximal antioncotic in each case.

We replicated the above published hormone treatments (5 groups of 30 rats) using the published doses and schedules, and found significantly reduced cancer incidence in every experiment. Blood was drawn three times from each group during the treatment and once after treatment for assay of rat AFP. These are in progress. If they show AFP present following all of the hormone challenges, it is likely that cancer inhibition is produced by a common inhibitor (AFP), and that subsequent effort for drug development should stress AFP and AFP analogs.

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## INTRODUCTION

In the US about 200,000 new breast cancer cases are diagnosed annually, and thousands die of the disease, often because the malignancy has become refractory to the treatment modalities that are now available. It is very important that we develop new therapeutic agents that are antioncotic by a mechanism different from those of current drugs. Our purpose is to develop such an agent that could be used as a first line treatment, solo or in combination with another agent, and that would have potential for use after other methods were no longer effective. Its use as a cancer preventive is possible. We have found that analogs of alphafetoprotein could be such an agent. They have anticancer activity utilizing a mechanism different from that of other drugs currently in use. Seeking new methods, several investigators have administered different pregnancy-associated hormones to carcinogen-treated rats and have observed significant reduction in breast cancer appearance. We think it is highly likely that such treatments elicit secretions of AFP, which in turn is the agent that inhibits cancer. Our experiments will determine whether the cancer inhibiting hormone treatments do in fact elicit the AFP which appears in the serum.

## BODY

### Materials and Methods

#### Animals

Female Sprague Dawley rats were obtained from Taconic Farms at age 34 days and were placed immediately on a controlled diet (Agway Pro-Lab 2000; Agway Corporation, Syracuse, NY). They were allowed free access to food and water, and maintained on a 12-hour light-dark cycle at a constant temperature (22°C) for the duration of the study.

#### Carcinogen Treatment

There were 30 rats in each experimental group (unless otherwise specified) to assure a 95% probability of detecting a difference between groups (ratios) of 40%, which was the difference seen for pregnancy (Grubbs et al A. 1983). NMU was obtained from the National Cancer Institute carcinogen repository (MRI, Inc. Kansas City, MO) and was dissolved in sterile physiological saline, (1% w/v), buffered to pH 5.0 with 3% acetic acid. At 50 days of age, rats were randomized, and while under anesthesia were numbered using an ear punch, and a single intracarotid injection of NMU, 50mg/kg body weight was administered.

#### Hormone Treatments

Each hormone treatment administered followed the doses and schedules specified in the publications being replicated.

##### $E_2 + P_4$

Ten days following carcinogen administration, 30 female rats received 20μg estradiol ($E_2$, obtained from Sigma Aldrich, St. Louis, MO ) plus 4mg

progesterone ($P_4$ ,obtained from Sigma Aldrich, St. Louis, MO) dissolved in sesame oil, daily, by 0.2ml subcutaneous injection, for 40 days.

**$E_3 + P_4$**

Ten days following carcinogen administration, 30 female rats received two individual subcutaneous silastic capsules (0.078 inch IDx 0.125 inch OD, 2cm long; Dow Corning, Midland, MI, USA) packed with 30mg $E_3$ (Sigma Aldrich, St. Louis, MO) and 30mg $P_4$ (Sigma Aldrich, St. Louis, MO), respectively. Each silastic capsule was implanted subcutaneously dorsally, on either side of the upper spine, while animals were under isoflurane anesthesia. Implants were left in place for 21 days to mimic pregnancy, and then removed.

**$E_3$**

Ten days following carcinogen administration, 30 female rats received a subcutaneous silastic capsule (0.078 inch IDx 0.125 inch OD, 2cm long; Dow Corning, Midland, MI, USA) packed with 30mg $E_3$ (Sigma Aldrich, St. Louis, MO). All silastic capsules were implanted subcutaneously dorsally, while animals were under isoflurane anesthesia. Implants were left in place for 21 days to mimic pregnancy, and then were removed.

**HCG**

Twenty-one days carcinogen administration, 30 female rats received an intra-peritoneal injection of 100IU lyophilized Human chorionic gonadotropin, (Sigma Aldrich, St. Louis, MO) reconstituted with de-ionized water, pH 7.2 , daily, for 60 days.

**Treatment with Pregnancy**

Ten days following carcinogen administration, 30 female rats were introduced to males (three females per male). Females stayed with males for 7 days, after which they were removed and separated. Twenty-one days later, 19 females bore litters, which were allowed to breast feed for 15 days. Females that did not become pregnant were excluded from the study.

**Mammary Carcinogenesis**

For tumor detection and monitoring of growth, animals were palpated twice weekly, starting at day 30 after NMU exposure for 123 days.

**Statistics**

The amount of inhibition provided by each hormone treatment was analyzed by comparing the percent incidence (number of rats bearing tumors) of the treatment with that of the NMU-only control (Fischer's Exact , $P<0.05$ was considered significant).

**Blood Samples**

Blood was drawn from each of the 6 groups of rats at 4 separate times; at the beginning, middle and end of the treatment, and once 14 days after the conclusion of the treatment.

Blood samples were collected from the tail vein. While rats were anesthetized under 3% isoflurane, 1ml of blood was drawn with a 25G. Blood was transferred to glass centrifuge tubes and allowed to clot, uncovered, on the bench at room temperature for 4 hours. Serum was drawn off of each sample and spun 3 times (1500rpm, for 10 min) after which supernatant was collected. Samples were stored in at -80 C until used in AFP assay.

**AFP Assays**

Detection in sera is by Western Blot, and for estimation of concentration employed a standard curve. Gels (12% Tris- HCl, BioRad, Hercules, CA) were run containing different concentrations of amniotic fluid, maternal rat serum from the same animals, and serum from animals that received no hormone treatment.Gels were transferred onto blots and after blocking non-specific sites with 5% milk buffer in 1x Tween Tris Buffered Saline (TTBS), blots were incubated overnight at 4°C with the primary antibody, goat anti rat AFP (Santa Cruz Biological, Santa Cruz, CA). Blots were rinsed with 1x TTBS and incubated with the secondary antibody, rabbit anti goat IgG HRP (Santa Cruz Biological, Santa Cruz, CA). Blots were rinsed again and then developed using Western Lightning Western Blot Chemiluminescence Reagent (Perkin Elmer, Boston, MA).

**Discussion**

Five challenges of carcinogen-treated rats with different anticancer agents have been preformed during the year. The outcomes in terms of breast cancer appearance over time are shown in Figures 1-5. Details of dosage amounts, routes and schedules appear in the legends to each figure. In each case the lower curve represents cancer appearance of animals in the tested group treated with an inhibitor, and the upper curve shows cancer appearance in a group of positive control rats (that received only carcinogen).

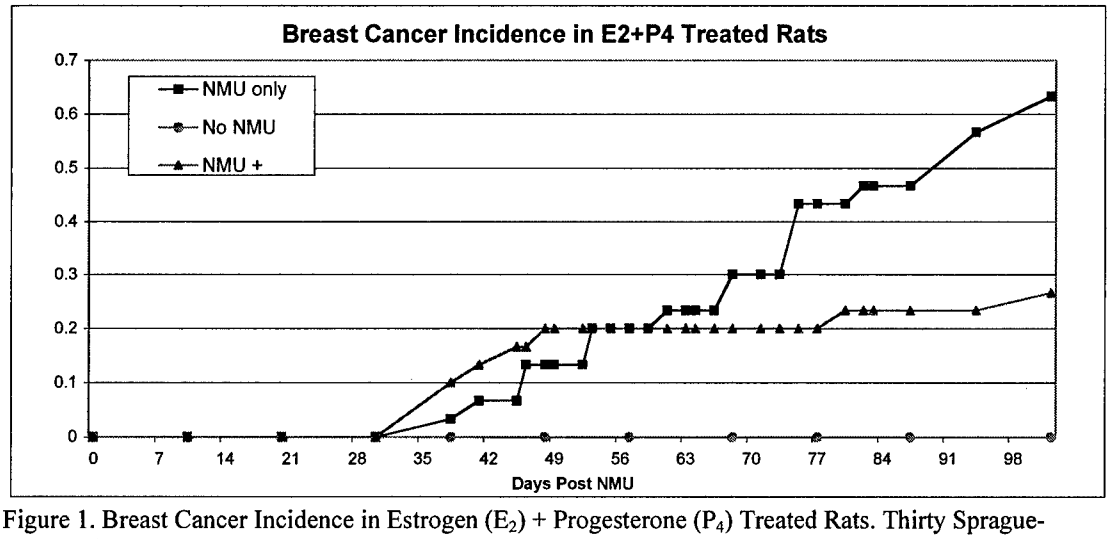


Figure 1. Breast Cancer Incidence in Estrogen ($E_2$) + Progesterone ($P_4$) Treated Rats. Thirty Sprague-Dawley female virgin rats received NMU (50mg/kg, intracarotid) at the age of 50 days. Ten days after, rats were administered a s.c. injection of 20μg $E_2$ + 4mg $P_4$ in sesame oil daily, for 40 days. By Day 102, $E_2$ + $P_4$ treatment decreased tumor incidence by 37% $p < 0.0036$. (Grubbs et al B, 1983).

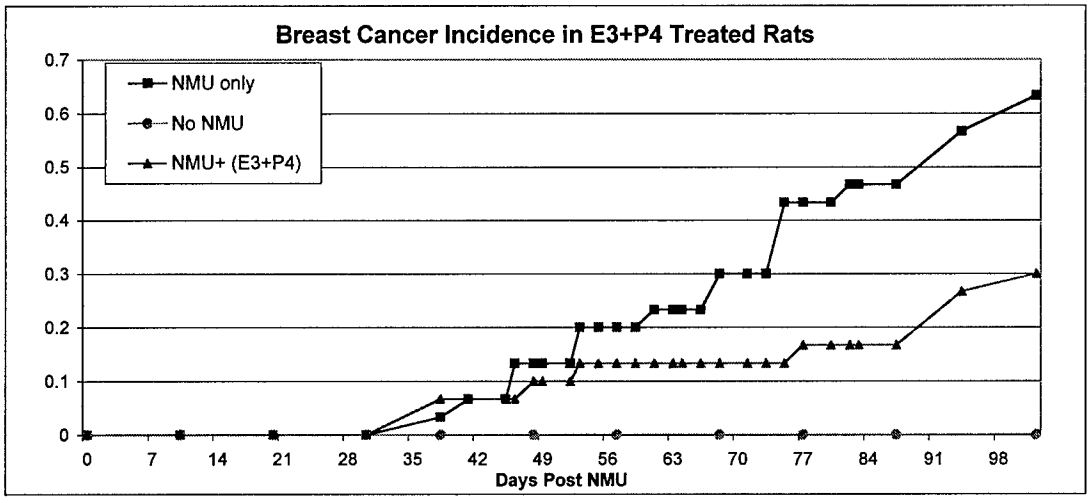


Figure 2. Breast Cancer Incidence in Estriol ($E_3$) + Progesterone ($P_4$) Treated Rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. Thirteen days after, rats received two subcutaneous silastic implants, containing 30mg estriol and 30mg progesterone respectively, which were left in place for 21 days, By Day 102, implants decreased tumor incidence by 33% $p< 0.008$. (Rajkumar et al, 2004).

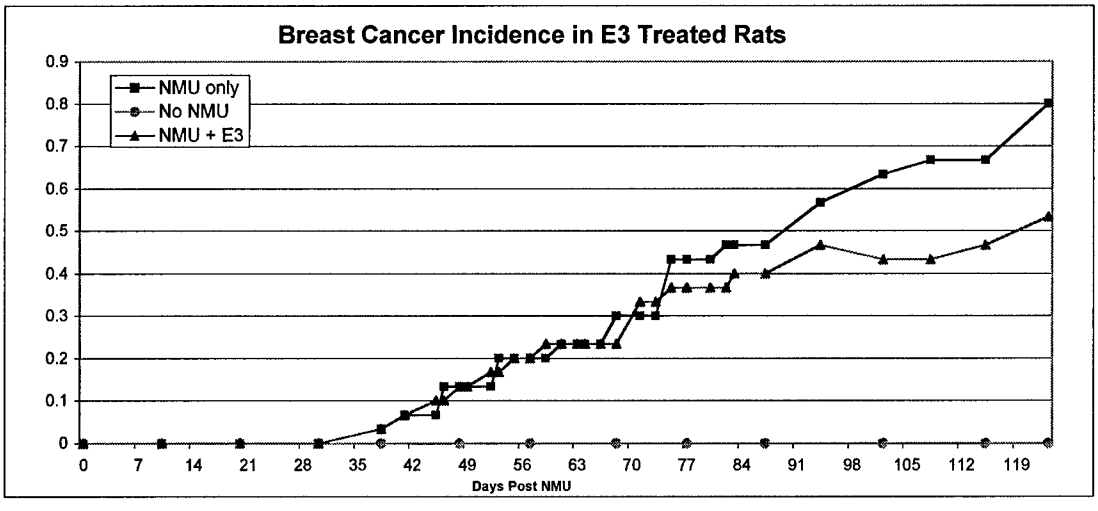


Figure 3. Breast Cancer Incidence in Estriol ($E_3$) Treated Sprague Dawley rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. Thirteen days after, rats received a single subcutaneous silastic implant containing 30mg estriol. Left under the skin for 21 days, By Day 102, implants decreased tumor incidence by 27% $p< 0.026$. (Rajkumar et al, 2004)

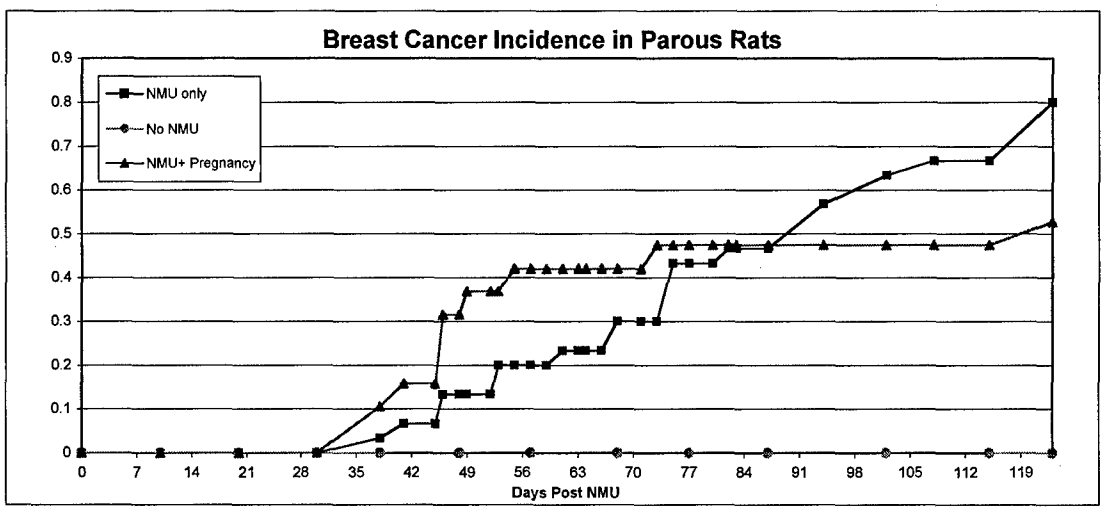


Figure 4. Breast Cancer Incidence in Mated Female Rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. Ten days after, 30 female rats were introduced to males (three females per male). Females stayed with males for 7 days, after which they were removed and seperated. Twenty-one days later, 19 females bore litters, which were allowed to breast feed for 15 days. Females that did not become pregnant were excluded from the study. By Day 123, pregnancy decreased tumor incidence by 28% $p < 0.035$. (Grubbs et al A, 1983).

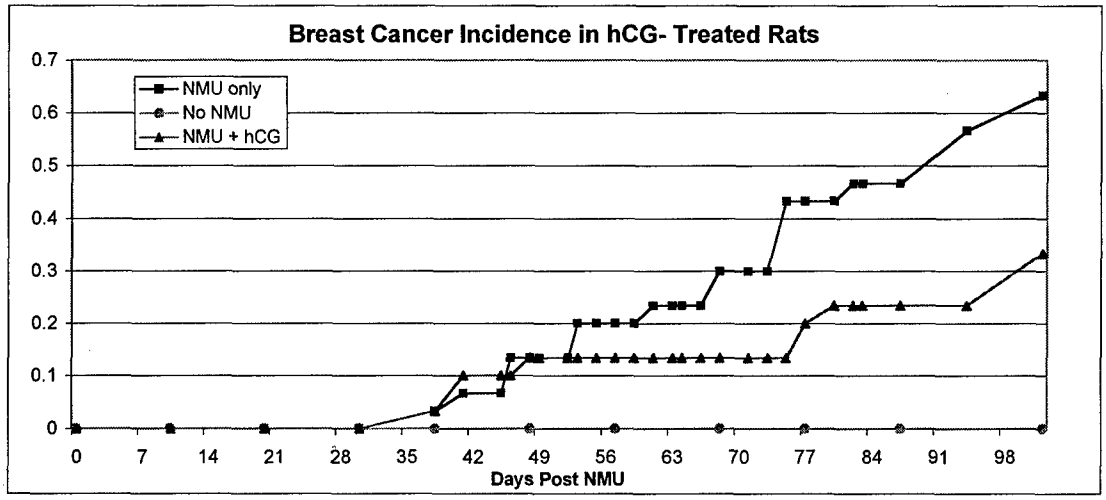


Figure 5. Breast Cancer Incidence in Human Chorionic Gonadotropin (hCG) Treated Rats. Twenty-one days after, rats were administered an i.p. injection of 100 IU hCG dissolved in de-ionized water daily, for a duration of 60 days. By Day 102, hCG treatment decreased tumor incidence by 30% $p < 0.0014$. (Russo et al, 1990).

For each of the groups, twice weekly palpations were continued for as long as was economically prudent or for a shorter time when statistically significant inhibition had been produced. On the final day of palpation the portion of rats that had tumors (i.e. "incidence") was compared with the (higher) incidence in the NMU-only controls. The statistical significance of their differences were determined and is shown in Table 1.

On day 102 the dimensions of each tumor were determined, and its volume estimated as an ellipsoid of revolution $V = \pi/6\ d^2\ D$, where d and D are the respective short and long diameters. For each group mean volume per rat was calculated (Table 1).

**Table 1. Treatment doses, schedules, and tumor incidence in NMU-treated Sprague Dawley female rats.**

| Treatment | No. of rats | Treatment Dose | Treatment Route | Treatment Start Date (Days Post NMU) | Treatment Duration (Days) | % Incidence/ Day | Number of Tumors Per Group Day 102 | Mean Number Tumors/Rat Day 102 | Mean Tumor Volume/Rat Day 102 ($mm^3$) |
|---|---|---|---|---|---|---|---|---|---|
| E2+P4 | 30 | 20μg $E_2$ + 4mg $P_4$ | s.c. injection | 13 | 40 | 26.6/ 102 ($p< 0.0036$)¥ | 10 | 0.33 | 2199 |
| E3+P4 | 30 | 30mg $E_3$+ 30mg $P_4$ | Silastic implant | 10 | 21 | 30.0/ 102 ($p< 0.008$) | 8 | 0.27 | 880 |
| E3 | 30 | 30mg | Silastic implant | 13 | 21 | 53.3/ 123 ($p< 0.026$) | 24 | 0.8 | 955 |
| Pregnancy | 19 | - | - | 10 | 21* | 52.6/ 123 ($p< 0.035$) | 28.42† | 0.98 | 2649 |
| hCG | 30 | 100 IU | i.p. injection | 21 | 60 | 33.3/ 102 ($p< 0.0014$) | 12 | 0.4 | 763 |
| Control | 30 | - | - | - | - | 63.3/ 102, 80.0/ 123 | 38 | 1.23 | 3504 |

* Normal gestation in a rat
† Normalized for group size of 30 animals
¥ P for differences from NMU control

Our goal is to determine the rat AFP concentration in the 20 serum samples that have been collected from each of the six groups. To perform these assays by ELISA, three anti- rat AFP antibodies were obtained from Santa Cruz (Santa Cruz, CA). However, no pair of them could be successfully developed into an ELISA. One of the antibodies that reacted strongly with the rat AFP now has been developed into a Western Blot assay for the antigen. Using this method, we obtained a standard curve that provides a minimum detectable AFP concentration of 1ng/ml, and this method is being employed for assay of rat sera.

## KEY RESEARCH ACCOMPLISHMENTS

1. Determined that $E_2$ + $P_4$ given to NMU-treated rats reduces mammary cancers by 37%.
2. Determined that $E_3$ + $P_4$ given to NMU-treated rats reduces mammary cancers by 33%.
3. Determined that $E_3$ given to NMU-treated rats reduces mammary cancers by 27%.
4. Determined that parity in NMU-treated rats reduces mammary cancers by 28%.
5. Determined that hCG given to NMU-treated rats reduces mammary cancers by 30%.
6. Established a Western Blot assay for the detection of rat AFP in the sera.

## REPORTABLE OUTCOMES

1. Presentation in the CORE Curriculum, Department of Pathology, Albany Medical College.

2. Mentoring medical student who has applied for and has been admitted to candidacy for the degree of Doctor of Medicine with Distinction in Research (MDDR).

## CONCLUSIONS

We have replicated the five published procedures for inhibiting appearance of carcinogen-induced breast cancer in rats. In each of the five, statistically significant inhibition was noted. Clearly the truncated curves in Figure 1-5 indicate that maintaining the animals for longer observation would show even greater degrees of inhibition. We have used larger groups of rats than did most of the previous studies in order to produce firm results.

We have collected a total of about 120 serum samples taken at times straddling the intervals of treatment for each group. The analysis will provide qualitative AFP data (it is present or absent) and quantitative data that would show the time course of AFP induction that each inhibition protocol could produce. Quantitative data that show differences in the maximum serum AFP concentration obtained by each group will relate directly to the degree of cancer inhibition seen.

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